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# Synthesis and optimization of a reactive oxygen species responsive cellular delivery system

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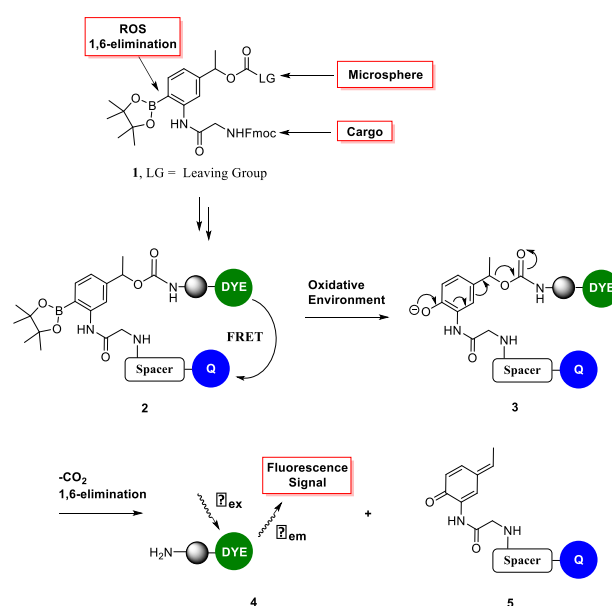
Reactive oxygen species play numerous roles in a number of pathological processes. Monitoring H<sub>2</sub>O<sub>2</sub> is a powerful tool for imaging and therapy of diseases wherein oxidative stress is involved. In particular, we report a specific application of functional microspheres as sensors of H<sub>2</sub>O<sub>2</sub>. Reactive oxygen species-responsive delivery systems were developed to detect *in vitro* peroxides thanks to the presence of a boronic ester which are readily cleaved with H<sub>2</sub>O<sub>2</sub>. This ROS-sensitive cleavable linker underwent a 1,6-elimination to disrupt of fluorescence resonance energy transfer by near-infrared fluorophores coupled such as Cy5.5/Cy7. This technology would allow real-time monitoring of therapeutic regimes (and their success), as well as optical detection of inflammation.

## 1. Introduction

In healthy cells and tissues, there exists a delicate balance between the generation of reactive oxygen species and radical generation and a cells antioxidant defences<sup>1,2</sup> with both important for normal metabolism, and regulation of cellular function.<sup>3,4</sup> Reactive oxygen species (ROS) cover a number of oxygen-based metabolites including superoxide anion radicals (O<sub>2</sub><sup>•−</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radicals (•OH). These species have been demonstrated to cause damage to various cellular components, effecting cellular proliferation and survival,<sup>5</sup> as well as being found in many hypoxic tissues. Perhaps counter intuitively compounds such as hydrogen peroxide are potentially more damaging to DNA than hydroxyl radicals, since their lower reactivity provides sufficient time for hydrogen peroxide to travel into the nucleus of a cell from its site of generation.<sup>6</sup>

H<sub>2</sub>O<sub>2</sub> imbalance has potential as a trigger for tissue directed delivery,<sup>7–10</sup> and has been extensively targeted with the synthesis of switch-on optical imaging probes.<sup>11–14</sup> Tsien developed a strategy where-by H<sub>2</sub>O<sub>2</sub> produced in the proximity of a cancer reacted with a so-called Activatable Cell Penetrating Peptide (ACPP) construct, inducing delivery of a labelled polyarginine scaffold into cells and with application in an *in vivo* inflammation model.<sup>15</sup>

The uptake of nano and microspheres (0.2–5 μm) has been successfully demonstrated in a broad range of cell types, including adherent and suspension cultures of primary cells. The broad applicability of this approach has been demonstrated with the “loading” of stem cells, giving rise to viable chimeras thus demonstrating their remarkable cellular biocompatibility.<sup>16–18</sup>



**Fig. 1** Fluorescence “turn on” ROS-responsive cleavable linker. Upper: Compound **1** shows the three major elements of the linker – namely (i) Its ability to be attached to a variety of cargos via Fmoc chemistry; (ii) A leaving group to allow conjugation to a cellular carrier; (iii) The cyclic boronate to allow peroxide mediated rearrangement to the phenol with subsequent 1,6-elimination.<sup>19</sup> The lower figure shows the mechanism of cleavage and the designed FRET system.

Here, ROS-cleavable linkers were designed, synthesised, loaded with a cargo and attached to microspheres, such that when these “carriers” entered cells undergoing oxidative stress cargo release was triggered. To allow evaluation of the cleavage of the ROS-sensitive linker, FRET-based probes were synthesised, as shown in Figure 1, where cleavage of the ROS-cleavable linker by H<sub>2</sub>O<sub>2</sub>

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perturbs the FRET system (consisting of the well-known FRET pair 5(6)-carboxyfluorescein ( $\lambda_{\text{Ex/Em}}$  488/528 nm)/Methyl red ( $\lambda_{\text{Abs}}$  480 nm) or the NIR cyanine dyes Cy5.5 ( $\lambda_{\text{Ex/Em}}$  675/694 nm)/Cy7 ( $\lambda_{\text{Ex/Em}}$  743/767 nm).<sup>20-22</sup> As a second example a cell carrier consisting of a polycationic lysine-like peptide,<sup>23</sup> which is resistant to proteolysis, non-toxic *in vivo* and has demonstrated a highly efficient cell entry profile<sup>24-34</sup> was used to confirm the versatility of ROS-sensitive linker.

This safety catch linker cleaves via the well-known oxidative rearrangement, which generates a phenol/phenolate which undergoes a 1,6-elimination reaction (as we described in 1997)<sup>35</sup> to liberate a fluorescent label or cargo.<sup>36</sup>

## 2. Experimental

### 2.1 Chemical and apparatus

All solvents and reagents were obtained from commercial suppliers and used without purification, unless otherwise stated. Microwave reactions were carried out using a Biotage Initiator instrument in sealed heavy-walled Pyrex tubes (Biotage AB, size 5 mL or 20 mL). Microspheres couplings were carried out on an Eppendorf ThermoMixer® comfort and washed with centrifugation in a Heraeus Pico 17 centrifuge.

Thin Layer Chromatography (TLC) was carried out using Merck silica gel 60 F254 (0.25 mm) foil-backed plates with visualisation by ultraviolet light. Flash chromatography was carried out using Silica 60 Å, particle size 35-70 micron under positive pressure.

High pressure liquid chromatography (HPLC) analyses were performed using the following eluents: A: H<sub>2</sub>O + 0.1% Formic Acid and B: MeOH + 0.1% Formic Acid. Analytical HPLC were performed on an Agilent 1100 analytical system with a Supelco Discovery® C18, 5 µm, 5 cm column coupled to a Polymer laboratories 100 ES evaporative light scattering detector (ELSD). HPLC grade eluents was employed, at a flow rate of 1 mL/min. The following method was used: eluents A and B, 5 to 95% B over 8 min, then 95% B for 1 min. Semi-preparative reverse phase-HPLC purifications were performed on an Agilent Technologies HP1100 Chemstation eluting on an Eclipse XDB-C18 column (9.4 x 250 mm, 5 µm), at a flow rate of 2 mL/min. Detection was carried out at 254 nm. The following gradient was used: 5 to 50 % A over 6 min, 50 to 100% B for 30 min. High Resolution Mass Spectrometry was measured in a Bruker MicroToF 2. Matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectra were performed using a Bruker Ultraflex Extreme MALDI TOFTOF instrument and analysed with the Bruker Daltonics flexAnalysis software. For peptide analysis, sinapic acid was used as a matrix and positive ion mass spectra were reported. Low resolution electrospray mass spectrum (ES/MS) was recorded on an Agilent Technologies LC/MSD 1100 Quadrupole Mass Spectrometer (QMS) with an electrospray ion source.

Nuclear Magnetic Resonance experiments were run in a Bruker Avance NMR Spectrometer 400 MHz or 500 MHz for small molecular weight compounds. Chemical shifts are reported as  $\delta$  in units of parts per million (ppm) relative to the indicated solvent. Multiplicities in <sup>1</sup>H NMR are reported as follows: *s* (singlet), *d* (doublet), *t* (triplet), *q* (quadruplet), *dd* (doublet of doublets), *ddd* (doublet of doublet of doublets), *m* (multiplet). Coupling constants

are reported as a *J* value in Hertz (Hz). The number of protons (*n*) for a given resonance is indicated as *n*H, and is based on spectral integration values.

Dynamic Light Scattering (DLS) and Zeta potential were measured on a Zetasizer Nano ZS ZEN 3500 in molecular biology grade water in a disposable sizing cuvette for size measurements or clear disposable zeta cuvettes for zeta potential measurements.

Fluorescence spectrophotometry was performed on a Synergy™ HT Multi-Mode Microplate Reader from BioTek. Reader control is via BioTek's Gen5™ Data Analysis Software.

Flow cytometry was performed on a BD FACS Aria® System using the BD FACS Diva® software. The data were analysed using the software Flowjo® 7.5.

### 2.2 Synthesis of ROS-cleavable linkers 1a/1b

**Synthesis of compound 7:** To a cold solution (-15°C) of Fmoc-protected glycine (7g, 23.36mmol) and 3-amino-4-bromoacetophenone **6** (5g, 23.54mmol) in pyridine (70mL), phosphoryl chloride (POCl<sub>3</sub>) (2.5mL, 26mmol) was added drop-wise. The mixture was stirred for 2 hours at -15°C and then allowed to warm to room temperature and was stirred for an additional hour. The reaction mixture was quenched with water (8mL). The solvents were evaporated under reduced pressure. The crude reaction mixture was purified by precipitation in DCM. Yield: 100%. HRMS: calcd for C<sub>25</sub>H<sub>21</sub>N<sub>2</sub>O<sub>4</sub>Br<sub>1</sub> 492.067920, found 492.067903. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.93 (*s*, 1H), 8.42 (*s*, 1H), 7.79 (*d*, *J* = 6.8, 3H), 7.62 (*d*, *J* = 4.9, 3H), 7.42 (*m*, 2H), 7.33 (*m*, 2H), 4.55 (*d*, *J* = 6.6, 2H), 4.27 (*t*, *J* = 6.4, 1H), 4.09 (*s*, 2H), 2.62 (*s*, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  197.10, 167.53, 143.56, 141.37, 137.18, 132.66, 127.86, 127.14, 124.89, 124.72, 121.75, 120.11, 67.52, 47.08, 45.81, 26.69.

**Synthesis of compound 8:** Compound **7** (4.5g) was dissolved in 82mL of 95% ethanol. Sodium borohydride (1.42g) was added and the mixture was stirred. The reaction was completed after 1h at room temperature. 500µL of ice-cold distilled water was slowly added by pipette to the reaction mixture. The mixture was transferred to a centrifuge tube and was extracted with dichloromethane (3 x 10mL) by mixing the mixture with a pipette. The solvent was removed on a rotary evaporator. No purification was necessary. Yield: 100%. HRMS: calcd for C<sub>25</sub>H<sub>23</sub>N<sub>2</sub>O<sub>4</sub>Br<sub>1</sub> 494.083570, found 494.082957. <sup>1</sup>H NMR (500 MHz, MeOD)  $\delta$  7.81 (*d*, *J* = 7.5, 2H), 7.68 (*t*, *J* = 9.5, 2H), 7.58 (*d*, *J* = 6.3, 1H), 7.35 (*ddd*, *J* = 25.0, 16.2, 8.8, 5H), 7.15 (*t*, *J* = 8.2, 1H), 4.81 (*dd*, *J* = 12.8, 6.4, 1H), 4.40 (*d*, *J* = 6.9, 2H), 4.25 (*t*, *J* = 6.9, 1H), 3.95 (*s*, 2H), 1.44 (*d*, *J* = 6.5, 3H). <sup>13</sup>C NMR (126 MHz, MeOD)  $\delta$  169.94, 168.96, 157.82, 150.99, 147.18, 143.89, 141.20, 128.90, 127.40, 126.78, 124.85, 121.44, 120.02, 119.56, 118.70, 117.40, 80.29, 69.31, 66.80, 43.94, 24.98.

**Synthesis of compound 9:** A dry reaction vessel was charged with Pd<sub>2</sub>(dba)<sub>3</sub> (13mg, 0.01eq) and tricyclohexylphosphine (PCy<sub>3</sub>, 20mg, 0.072eq) and flushed with nitrogen. 1mL of dioxane was added and the resulting mixture was then stirred for 15 minutes at room temperature. A 100mL flask containing 500mg of **8** was charged with bis(pinacolato)diboron (258.7mg, 1.0eq) and KOAc (142mg, 1.5eq), flash evacuated and backfilled with nitrogen. 4mL of dioxane was added and the resulting mixture stirred. The contents of the two reactions were mixed and bubbled with nitrogen to reduce oxygen. The resulting mixture was stirred at reflux (80°C) overnight. The crude product was purified by column

chromatography with 20% MeOH in DCM. Yield: 50%. HRMS: calcd for  $C_{31}H_{35}N_2O_6B_1$  542.258270, found 542.257887.  $^1H$  NMR (400 MHz, MeOD)  $\delta$  7.83 (d,  $J$  = 7.6, 2H), 7.71 (d,  $J$  = 7.3, 2H), 7.58 (d,  $J$  = 7.6, 1H), 7.44 - 7.33 (ddd,  $J$  = 32.6, 14.6, 7.4, 4H), 7.26 (dd,  $J$  = 10.1, 6.2, 2H), 4.84 (m, 1H), 4.43 (d,  $J$  = 6.8, 2H), 4.26 (t,  $J$  = 6.8, 1H), 4.18 (s, 2H), 1.44 (d,  $J$  = 6.4, 3H), 1.31 (s, 12H).  $^{13}C$  NMR (100 MHz, MeOD)  $\delta$  172.26, 169.70, 143.83, 141.24, 133.13, 132.99, 127.44, 126.80, 124.80, 119.56, 69.05, 66.94, 42.84, 30.41, 29.49, 29.38, 29.08, 24.76.

**Synthesis of ROS cleavable linker 1a (3-[Fmoc-Gly]-4-[(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)]phenyl]ethyl 2,5-dioxo-1-pyrrolidinyl ester carbonic acid):** To stirred solution of **9** (200mg, 0.37mmol) in dry MeCN/DCM (7.5mL),  $N,N'$ -disuccinimidyl carbonate (142.17mg, 0.555mmol) was added at room temperature under a  $N_2$  atmosphere. To the cloudy suspension, triethylamine (154 $\mu$ L, 1.11mmol) was added, and after a couple of minutes the reaction mixture turned clear. The reaction was completed after 4h. The solvent was removed in vacuo. The residue was dissolved in 6mL  $CH_2Cl_2$ , and washed with 3 x 3mL saturated  $NaHCO_3$ . The organic phase was washed with 4mL brine and dried with anhydrous  $MgSO_4$ , and the solvent was removed in vacuo. The crude product was purified by column chromatography eluting with 70% EtOAc in hexane Yield: 30%. HRMS: calcd for  $C_{36}H_{39}N_3O_{10}B_1$  684.345070, found 684.345500.  $^1H$  NMR (400 MHz, MeOD)  $\delta$  7.96 (d,  $J$  = 1.5, 1H), 7.82 (d,  $J$  = 7.4, 2H), 7.71 (m, 2H), 7.58 (d,  $J$  = 8.2, 1H), 7.46 - 7.30 (m, 4H), 7.16 (m, 2H), 4.82 (m, 1H), 4.46 (d,  $J$  = 6.7, 2H), 4.27 (t,  $J$  = 7.5, 1H), 3.98 (s, 2H), 1.44 (d,  $J$  = 6.6, 3H), 1.30 (s, 4H), 1.22 (s, 12H).  $^{13}C$  NMR (126 MHz, MeOD)  $\delta$  169.94, 168.86, 157.82, 147.18, 143.89, 141.20, 140.32, 128.90, 128.39, 127.40, 126.78, 124.85, 121.07, 119.53, 118.70, 117.15, 80.29, 69.31, 66.80, 43.94, 28.98, 23.65.

**Synthesis of ROS cleavable linker 1b (3-[Fmoc-Gly]-4-[(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)]phenyl]ethyl 4-nitrophenyl ester carbonic acid):** Benzyl alcohol derivative **9** (0.4g, 0.74mmol) was dissolved in 7.4mL of a dry mix of DCM/THF (1:1) under  $N_2$ . Pyridine (anhydrous) (0.12mL, 1.48mmol) was added followed by 4-nitrophenyl chloroformate (0.16g, 0.81mmol) and the reaction was allowed to stir at room temperature for 12h. The reaction was diluted with EtOAc and washed with 1M HCl followed by saturated  $NaHCO_3$ . The organic layer was dried over  $MgSO_4$ , filtered and concentrated. Compound was purified on a silica gel column eluting

with 5% EtOAc in hexane. Yield: 60%. HRMS: calcd for  $C_{38}H_{38}N_3O_{10}B_1$  707.264480, found 707.264750.  $^1H$  NMR (500 MHz, MeOD)  $\delta$  8.00 (s, 1H), 7.80 (t,  $J$  = 8.3, 3H), 7.70 (d,  $J$  = 7.3, 3H), 7.59 (d,  $J$  = 8.3, 1H), 7.44 - 7.27 (m, 6H), 7.12 (d,  $J$  = 8.0, 1H), 5.84 (dd,  $J$  = 12.8, 6.2, 1H), 4.46 (d,  $J$  = 6.7, 2H), 4.27 (t,  $J$  = 6.6, 1H), 3.99 (s, 2H), 1.52 (d,  $J$  = 7.6, 3H), 1.27 (s, 12H).  $^{13}C$  NMR (126 MHz, MeOD)  $\delta$  171.93, 169.25, 143.82, 142.43, 141.24, 135.36, 132.39, 127.43, 126.80, 124.80, 119.58, 83.26, 74.45, 71.76, 66.90, 46.98, 35.00, 34.52, 28.98, 28.45, 26.44, 26.34, 25.86, 25.84, 23.96, 23.94, 23.65, 21.06.

### 2.3 Synthesis of ROS-responsive delivery systems

**Synthesis of Polymeric Microspheres:** 200nm amino functionalised microspheres were synthesized following the methodology developed previously.<sup>37-39</sup>

**Synthesis of the probe 15:** The dual-labelled 200 nm microspheres (**15**) and Cy5.5 labelled-microspheres (**positive control**) were prepared using an orthogonal Fmoc/Dde-based strategy following the solid phase protocol described in the Electronic Supporting Information.<sup>37-39</sup> The colloidal stability of the microspheres was measured by Phase Analysis Light Scattering in the Zetasizer Nano ZS. It was observed that the values of zeta potential change significantly after each step.

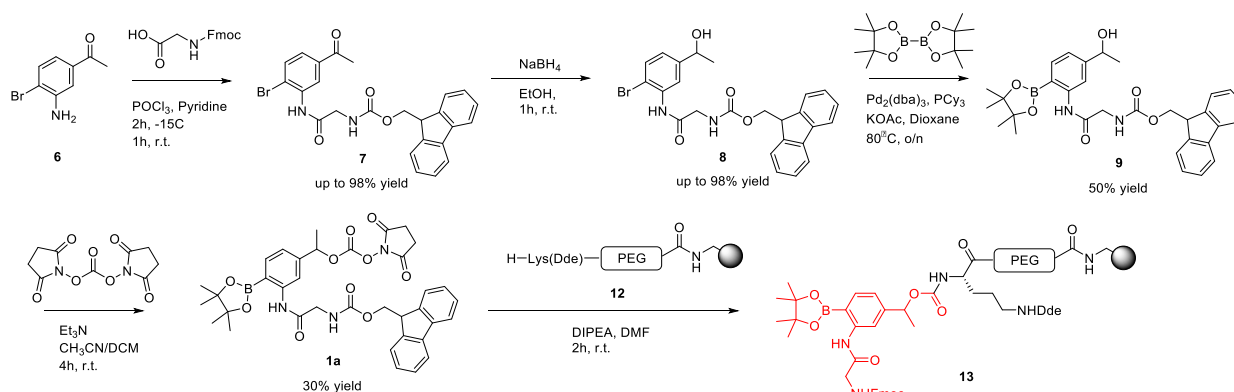
**Synthesis of ROS-responsive cell penetrating peptide mimetic (CPPM) probes 22 and 23:** A highly-optimised microwave-based solid-phase strategy was used to synthesise the cell penetrating peptides.<sup>24</sup> An aminomethyl-PS (2% DVB) resin functionalised with a Rink amide type linker was used for the synthesis of the peptides. An Fmoc-based reaction scheme was followed to obtain the desired probes following the methodology developed before<sup>23</sup> (see full details in the Electronic Supporting Information).

### 2.4 ROS-cleavable linker detection assay

ROS cleavable linker **1b** (1.4mg) was dissolved in 2mL of 2M hydrogen peroxide solution (1mM), and after 5 mins was analysed by HPLC and LCMS to determine the cleavable products. ROS cleavable linker **1b** was dissolved in 2mL of water at 1mM as a control.

### 2.5 Sensitivity test

ROS-responsive cell penetrating peptide probes **22** and **23** (20 $\mu$ M) were incubated for 10 mins with  $H_2O_2$  (0-40mM) in PBS (1mL) and fluorescence emission at  $\lambda_{ex/em}$  485/528 nm for FAM/MR and 590 /



**Scheme 1** Successful synthetic route to the ROS-cleavable linker.

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645 nm for Cy5.5/Cy7 was monitored ( $n = 3$ ) in a BioTek microplate reader. Error is  $\pm$  standard deviation.

ROS-responsive microspheres probe **15** and Cy5.5 labelled-microspheres (**positive control**) (85  $\mu\text{g/mL}$ ), were incubated with  $\text{H}_2\text{O}_2$  (40 mM) in PBS (1 mL) and fluorescence emission at  $\lambda_{\text{ex/em}}$  590/645 nm for Cy5.5/Cy7 was monitored ( $n = 3$ ) by a BioTek microplate reader. Also, the fluorescence was measured at  $\lambda_{\text{ex/em}}$  482/690 nm, using a BD FACS Aria flow cytometer. Fluorescence was evaluated as the mean fluorescence intensity (MFI) with 1,000 events per sample plotted in two-dimensional dot plots based on forward and side scattering (SSC-H vs. FSC-H) to gate microsphere populations. The data were analysed using the software Flowjo® 7.5. Error is  $\pm$  standard deviation.

### 2.6 Cellular assays

Cell experiments were performed in a HeraCell 150 incubator and carried out in a Herasafe KS 18 class II negative-flow cabinet from Heraeus. Cells were cultured in DMEM containing high glucose (4.5 mg/mL) supplemented with 4 mM glutamine, 100 units/mL penicillin, 50 mg/mL streptomycin, and 10% FBS. The cells were maintained in a humid chamber at 37°C in an atmosphere of 5%

$\text{CO}_2$  in T75 cell culture flasks until 70% confluency. RAW 264.7 macrophage cells in their complete growth medium were transferred (100,000 cells/well) to 24-well plates. To activate  $\text{H}_2\text{O}_2$  production,  $\text{PbCrO}_4$  was added (150  $\mu\text{M}$ ) and incubated during 20 mins. After the removal of media, ROS-responsive cell penetrating peptide probes **22-23** and CPPM positive control (5  $\mu\text{M}$ ) or ROS-responsive microspheres probe **15** and Cy5.5 labelled-microspheres positive control (85  $\mu\text{g/mL}$ ) were incubated overnight. Then, cells were washed twice with PBS and detached with trypsin/EDTA, harvested with 2% fetal bovine serum (FBS) in PBS (supplemented with Trypan Blue (0.04%) for FITC), analysing using a BD FACS Aria flow cytometer. Cellular fluorescence of cells was analysed in a Zeiss Axiovert 200M pseudo confocal microscope with a 100 W Hg lamp or in a Leica SP5 Confocal (GFP and RFP channel).

## 3. Results and discussion

The ROS-cleavable linker was synthesised in 4 steps following the approach shown in Scheme 1.

**Table 1** Reagents and reaction conditions used for optimisation of the synthesis of compound **9**.

Reagents	Ratios ( <b>8</b> ) <sup>a</sup>	Solvent/Conc. of substrate ( <b>8</b> )	Conditions	Yield (%)
$\text{PdCl}_2(\text{dppf})$ KOAc	0.03 1.5	Dioxane 0.1 M	24h 80°C	14%
$\text{PdCl}_2(\text{dppf})$ KOAc	0.03 1.5	Dioxane 0.1 M	48h 80°C	19%
$\text{PdCl}_2(\text{dppf})$ $\text{Et}_3\text{N}$	0.03 1.5	Dioxane 0.1 M	3h 100°C	14%
$\text{Pd}_2(\text{dba})_3$ $\text{PCy}_3$ KOAc	0.03 0.07 1.5	Dioxane/ $\text{H}_2\text{O}$ (1:3)/0.2 M	3h 80°C microwave heating	-
$\text{Pd}_2(\text{dba})_3$ $\text{PCy}_3$ KOAc	0.03 0.07 1.5	DMF/ $\text{H}_2\text{O}$ (1:3)/0.2 M	3h 80°C microwave heating	-
$\text{Pd}_2(\text{dba})_3$ $\text{PCy}_3$ KOAc	0.03 0.07 1.5	Dioxane 0.2 M	6h 80°C	31%
$\text{Pd}_2(\text{dba})_3$ $\text{PCy}_3$ KOAc	0.03 0.07 1.5	Dioxane bubbled 0.2 M	6h 80°C	50%

**Table 2** Reagents and reaction conditions used in the optimisation of the synthesis of the ROS-cleavable linkers **1a/1b**.

Reagents	Ratios ( <b>9</b> )	Solvent/Conc. of substrate ( <b>9</b> )	Conditions	Yield (%)
BTC TEA	2 1	DCM/0.1 M	1h 0°C 6h r.t.	-
BTC Pyridine	2.3 13.5	DCM/0.1 M	2h	-
BTC	0.1	DMF/ $\text{CH}_3\text{CN}$ (1:1)/0.1 M	24h	-
CDI DMAP	4 0.5	DMF/0.2 M	24h	-
$4\text{NO}_2\text{PhCOCl}$ TEA	1.1 2	THF/0.1 M	2h	-
$4\text{NO}_2\text{PhCOCl}$ Pyridine	2 3	DCM/0.8 M	3h	10%
DSC $\text{Et}_3\text{N}$	1 2	$\text{CH}_3\text{CN}/\text{DCM}$ (1:1)/0.4 M	4h	30% ( <b>1a</b> )
$4\text{NO}_2\text{PhCOCl}$ Pyridine	2 3	DCM/THF (7:3)/0.4 M	o/n	60% ( <b>1b</b> )

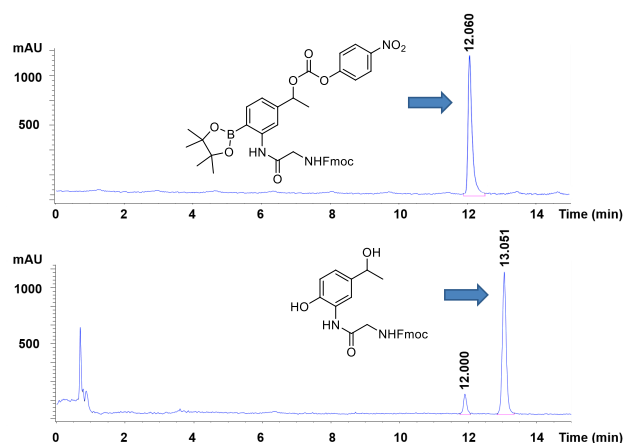
<sup>a</sup> Bis(pinacolato)diboron was used at 1:1 equivalents according to compound **8** in all cases.

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Compound **7** was synthesised from 3-amino-4-bromoacetophenone **6** by the amide coupling of Fmoc-Gly-OH. Presumably due to the poor nucleophilic behaviour and steric bulk of the bromoaniline, compound **7** was only obtained using the acid chloride of Fmoc-Gly-OH, generated using phosphoryl chloride (5.8% w/v) in pyridine for 2h at -15°C.

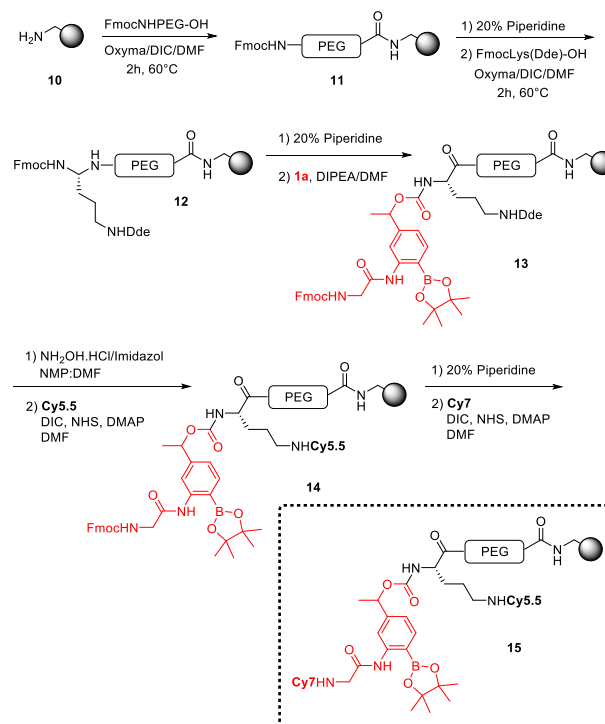
Following successful amide bond formation, the ketone was simply reduced by NaBH<sub>4</sub> to give the alcohol **8**. Palladium mediated conversion of the bromide to the boronic ester was problematic and took extensive optimisation (Table 1). The use of the ligand tricyclohexylphosphine and the palladium catalyst Pd<sub>2</sub>(dba)<sub>3</sub> gave the desired product in 50% yield.

The next step was the activation of the secondary alcohol group. Several strategies were tried, including triphosgene bis(trichloromethyl) carbonate (BTC), 4-nitrophenyl chloroformate, 1,1'-carbonyldiimidazole (CDI), and N,N'-disuccinimidyl carbonate (DSC) (Table 2).



**Fig. 2** HPLC chromatograms (detection at 254 nm) of Upper the linker (**1b**), Lower its cleavage products following treatment with peroxide.

To test whether the linker was cleaved by H<sub>2</sub>O<sub>2</sub>, linker **1b** (3-[Fmoc-Gly]-4-[(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)]phenyl]ethyl 4-nitrophenyl ester carbonic acid) was treated with hydrogen peroxide and analysed by HPLC/LCMS. This clearly showed (Figure 2), the generation of the phenol derivative (2-[Fmoc-Gly]-4-hydroxyethylphenol), a product of the 1,6-elimination reaction generated by the oxidation of the boronic acid in the classical boronic acid to phenol rearrangement.

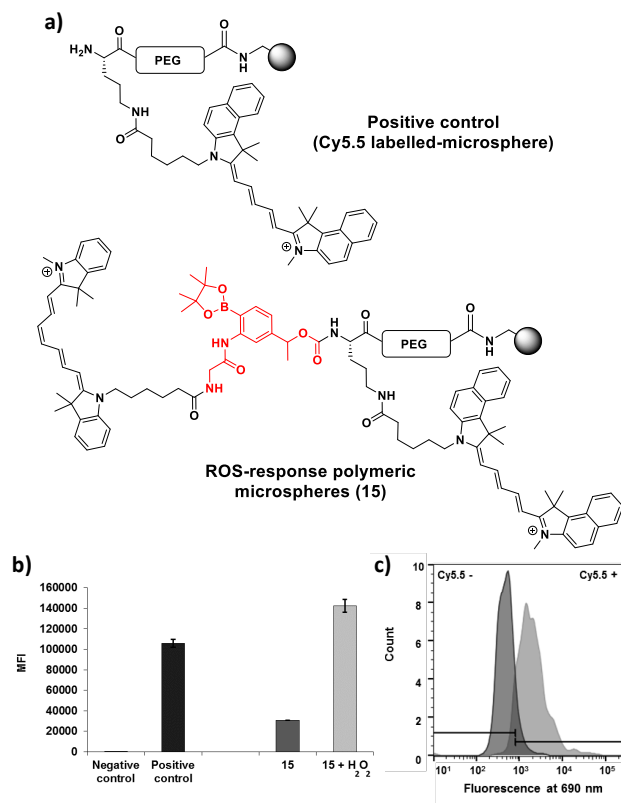


**Scheme 2** General synthesis for the generation of ROS-responsive microspheres (**15**).

Using 4-nitrophenylchloroformate or N,N'-disuccinimidyl carbonate, the linker was successfully activated to give the 4-nitrophenyl and succinimidyl carbonates respectively. However, 4-nitrophenol was not a good enough leaving group to enable coupling to the amino derivatised microspheres, whereas the N-hydroxysuccinimide was. This then allowed solid-phase orthogonal chemistry (based on Dde and Fmoc protecting group chemistry)<sup>36</sup> to allow coupling of peptide cargos/dyes to the microspheres (Scheme 2).

The polymeric microspheres were synthesized by an emulsifier-free emulsion polymerisation<sup>16</sup> where vinylbenzylamine hydrochloride salt (VBAH) acts as the emulsifier and is copolymerized with styrene along with p-divinylbenzene (DVB) as a cross-linking agent (see ESI). A Cy5.5/Cy7 FRET pair was used to allow evaluation of cellular-uptake efficiency and monitoring of ROS-production. The dual-labelled 200 nm microspheres (loading 0.2 mmol/g) were prepared using an orthogonal Fmoc/Dde-based strategy. Thus Fmoc-PEG-OH was coupled onto the microspheres followed by coupling of Fmoc-Lys(Dde)-OH and attachment of the linker **1a**.<sup>18</sup> The Dde protecting group was selectively removed<sup>36</sup> followed by the addition of Cy5.5. The dye Cy7 was coupled following Fmoc cleavage (see Scheme 2).



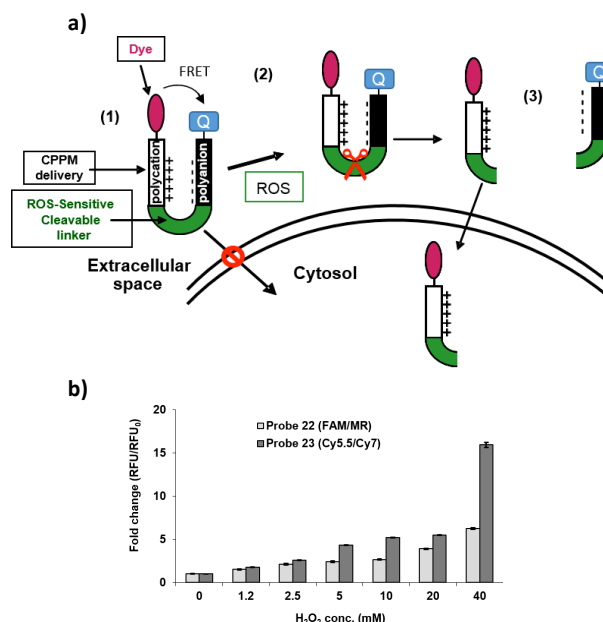


**Fig. 3** (a) Design of ROS-responsive Polymeric Microspheres (**15**) as a FRET-based probe attached to the ROS-cleavable linker (**1a**), which is cleaved by oxidative conditions perturbing the FRET system (consisting of NIR cyanine dyes Cy5.5 ( $\lambda_{\text{ex}}/\lambda_{\text{em}}$  675/694 nm)/Cy7 ( $\lambda_{\text{ex}}/\lambda_{\text{em}}$  743/767 nm)). (b) Flow cytometry analysis of fluorescence intensity. The ROS-responsive polymeric microspheres (**15**) showed > than 3-fold fluorescence increase after 10 min incubation with H<sub>2</sub>O<sub>2</sub> (40 mM). (c) Histograms of the microspheres probe **15** before (dark grey) and after (light grey) the addition of H<sub>2</sub>O<sub>2</sub> ( $\lambda_{\text{ex}}/\lambda_{\text{em}}$  482/690 nm), using a BD FACSARIA flow cytometer. Fluorescence was evaluated as the mean fluorescence intensity (MFI) with 1,000 events per sample plotted in two-dimensional dot plots based on forward and side scattering (SSC-H vs. FSC-H) to gate microsphere populations. The data were analysed using the software Flowjo® 7.5.

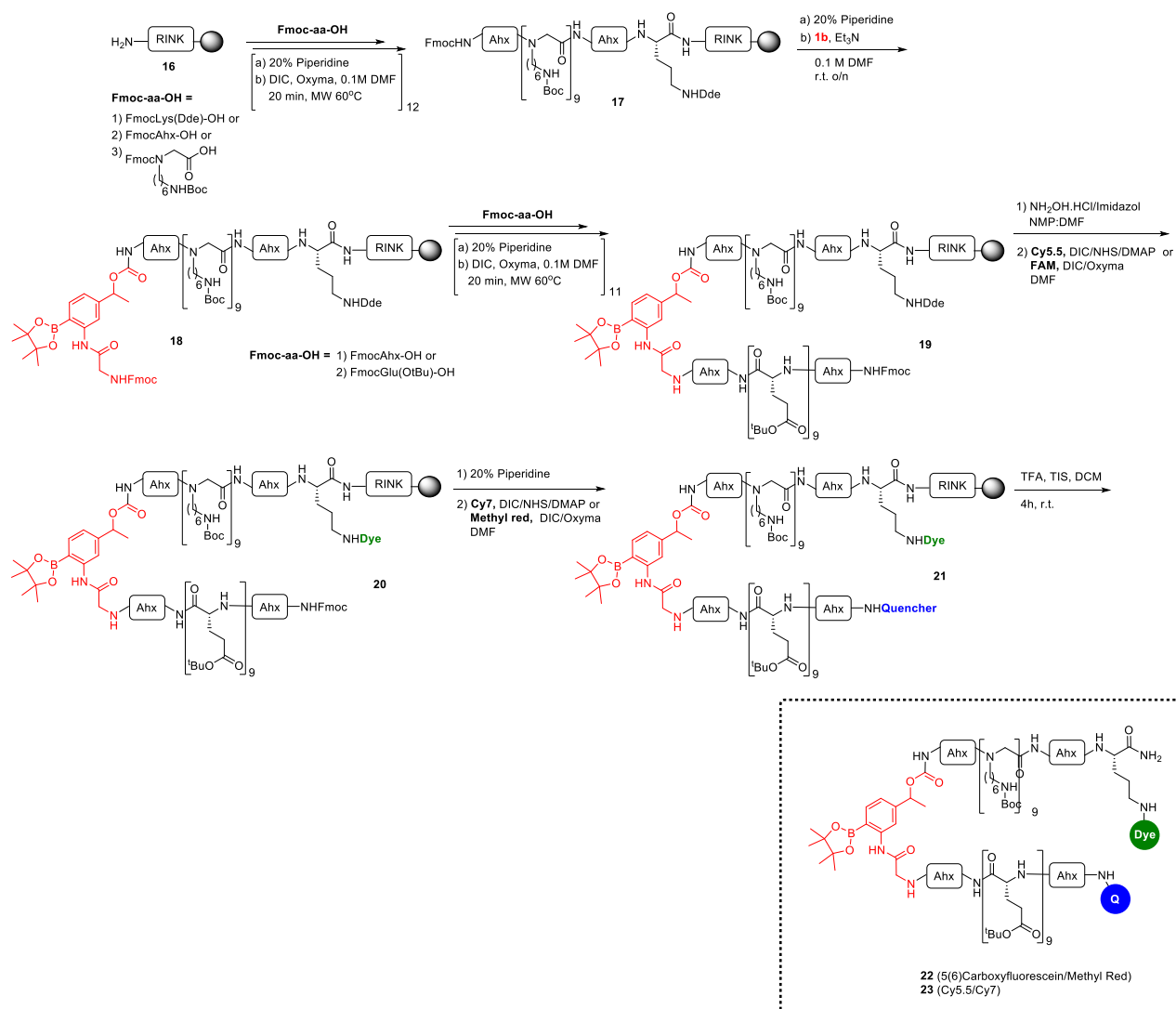
To test the efficiency of ROS-responsive microspheres (**15**) for the monitoring of oxidative species in solution, a flow cytometry assay was performed on the beads with probe **15** incubated with H<sub>2</sub>O<sub>2</sub> (see figure 3). This showed a >3-fold Cy5.5-fluorescence increase and confirms Cy5.5 fluorescence quenching (Cy5.5/Cy7 FRET) with disruption of this FRET pair through cleavage of the ROS-linker. Another approach to confirm the efficiency of the ROS-sensitive linker was designed using a cell penetrating peptide as a carrier system. This consisted of the polycationic N-alkylglycine peptide<sup>24-34</sup> connected to the ROS-cleavable linker **1b** (which showed high coupling efficiency to the Rink amide resin than **1a**), but with cellular uptake blocked due to interaction with a polyanionic chain

(L-Glu<sub>9</sub>) analogous to the system of Tsein (for synthesis details see ESI). The ROS-responsive cell penetrating peptides **22-23** were synthesized on a Rink amide-functionalised aminomethyl polystyrene resin using an Fmoc/tBu-based strategy and labeled with the fluorophores 5(6)-carboxyfluorescein or Cy5.5 and the corresponding quenchers methyl red and Cy7 (see Scheme 3).<sup>23,24</sup> After deprotection and cleavage from the resin with TFA/TIS, the ROS-responsive Activatable Cell Penetrating Peptides were purified by preparative HPLC and analysed with structures confirmed by MALDI-TOF MS.

The efficiency of the release of the cargos was evaluated in solution assays with monitoring of the fluorescence increase when probes **22** and probe **23** were incubated with increasing concentrations of H<sub>2</sub>O<sub>2</sub> (Figure 4b) and showed a 6-fold fluorescence increase for the peptide **22** carrying the FAM/MR pair and a 15-fold fluorescence increment for the peptide **23** what contained the Cy5.5/Cy7 couple. RAW 264.7 macrophage cells (mouse leukemic macrophage) are a cell line established from a tumor induced by Abelson murine leukemia virus. They are capable of producing H<sub>2</sub>O<sub>2</sub> upon stimulation with various inorganic/organic molecules.<sup>40,41</sup>



**Fig. 4** (a) ROS-responsive cell penetrating probes (**22** and **23**) – The Reactive Oxygen Species – activatable cell penetrating strategy. (a) (1) Blocked cellular uptake of the delivery system due to neutralisation / interaction with the polyanionic chain; (2) Selective extracellular cleavage of the linker by the reactive oxygen species and (3) the liberated polycationic peptide linked to the dye can enter the cell. (b) Analysis of the cell penetrating peptides **22** and **23** (at 20  $\mu$ M) with differing concentrations of hydrogen peroxide (measured 10 min after addition of H<sub>2</sub>O<sub>2</sub>): Peptide **22**,  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  485/528 nm or **23**,  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  590/645 nm (Cy5.5  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  675/694 nm and Cy7  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  743/767 nm).



**Scheme 3** General synthesis of ROS-responsive cell penetrating peptides (**22** and **23**).

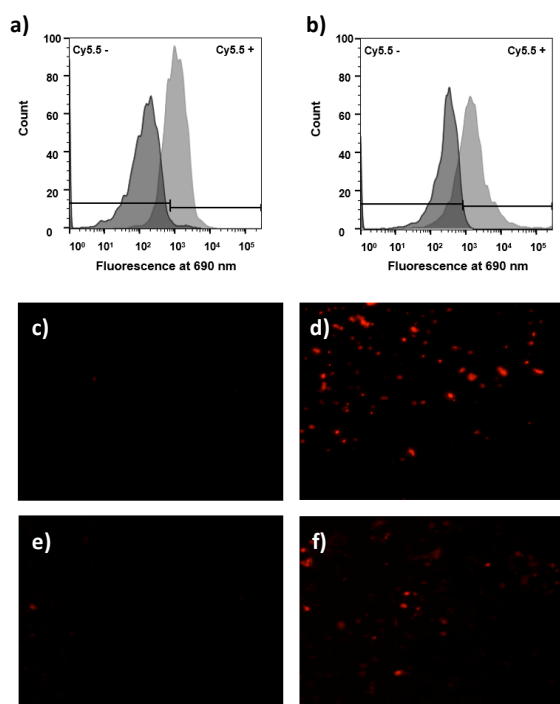
The probes **15** and **23** were incubated with either oxidative or non-oxidative environments, with RAW macrophage cells and cell fluorescence analysed by flow cytometry and fluorescence microscopy. It has been shown that  $\text{PbCrO}_4$  can be phagocytized by cells and can accumulate within vacuoles in the cytoplasm.  $\text{Cr(VI)}$  compounds undergo a complex metabolic reducing pathway, which generates a variety of reactive forms of chromium and ROS, and generate hydroxyl radicals ( $\cdot\text{OH}$ ) from  $\text{H}_2\text{O}_2$  via a Fenton-like reaction. Also, during the cellular  $\text{Cr(VI)}$  reduction process, molecular oxygen is consumed to generate superoxide anion radicals ( $\text{O}_2^{\cdot-}$ ) and  $\text{H}_2\text{O}_2$ .<sup>42</sup>

When RAW cells were treated with the ROS-responsive cell penetrating microspheres (probe **15**) (85  $\mu\text{g/mL}$ ) and were stimulated with  $\text{PbCrO}_4$  (150  $\mu\text{M}$ ) the flow cytometric histograms showed >20-fold more fluorescence for ROS-production stimulated cells (Fig. 5a). Semi-confocal microscopy confirmed that the fluorescence signal was far greater after linker cleavage (Fig. 5c-d), and confirming the results obtained by flow cytometry.

It was explored whether the ROS-responsive cell penetrating peptide (probe **23**) (5  $\mu\text{M}$ ) could be used to detect  $\text{H}_2\text{O}_2$  in the cellular environment as observed previously using the ROS-responsive microspheres. Probe **23** was incubated with RAW cells with/without  $\text{PbCrO}_4$  and analysed by flow cytometry and



fluorescence microscopy. In this case, the flow cytometric histograms showed a 6-fold fluorescence increment for cells treated with  $\text{PbCrO}_4$  (Fig. 5b), corroborating the successful cleavage of the ROS-sensitive linker when the  $\text{H}_2\text{O}_2$  level increased.



**Fig. 5** Assessment of the ROS-sensitive linker in probe **15** and **23** in live RAW cells showing cleavage by  $\text{H}_2\text{O}_2$ , as demonstrated by FACS analysis and microscopy. Histograms of RAW cells incubated with (a) ROS-sensitive microspheres probe **15** (85 µg/mL) (dark grey) and probe **15** (85 µg/mL) +  $\text{PbCrO}_4$  (150 µM for 20 min) (light grey) or incubated with (b) ROS-sensitive cell penetrating peptide probe **23** (5 µM) (dark grey) and probe **23** (5 µM) +  $\text{PbCrO}_4$  (150 µM for 20 min) (light grey). (c-d and e-f) Zeiss Axiovert 200M pseudo confocal microscope images of RAW cells analysed under a Cy5 filter ( $\lambda_{\text{ex}}/\lambda_{\text{em}}$  640/690 nm), with a x20 objective (c) after incubation with **15** (85 µg/mL) and (d) after incubation with **15** (85 µg/mL) +  $\text{PbCrO}_4$  (150 µM for 20 min); (e) after incubation with **23** (5 µM) and (f) after incubation with **23** (5 µM) +  $\text{PbCrO}_4$  (150 µM for 20 min). Red fluorescence dots are from Cy5.5 “turned on” by hydrogen peroxide cleavage of the ROS-sensitive linker in the ROS-sensitive microspheres probe **15** (upper) and the ROS-sensitive cell penetrating peptide probe **23** (lower) inside the cytoplasm of RAW macrophage cells.

## Conclusions

In conclusion an efficient route to synthesize a ROS-cleavable linker that is sensitive to  $\text{H}_2\text{O}_2$  has been developed. This linker allowed the design of ROS-responsive delivery systems able to liberate cargos selectively into the cellular cytoplasm in an oxidative environment. The synthesis of a ROS-responsive polymer delivery system that detects ROS inside cells allowed the release of the cargos in the cellular cytoplasm. Cell penetrating peptide mimetics were

designed with penetrating properties disrupted through a hairpin structure with a FRET pair, and the cleavage of the linker permits the internalization of labelled-peptide. In live cells, fluorescence increases were detected by flow cytometry, confirming the potential of these ROS-responsive delivery systems for imaging of oxidative environment. Based on these studies, phagocytic cells (macrophages) which generate high levels of  $\text{H}_2\text{O}_2$ , could be monitored thanks to the efficient cleavage of this ROS-sensitive linker. Importantly, a similar targeting mechanism could be further used for directed delivery of therapeutic cargos.

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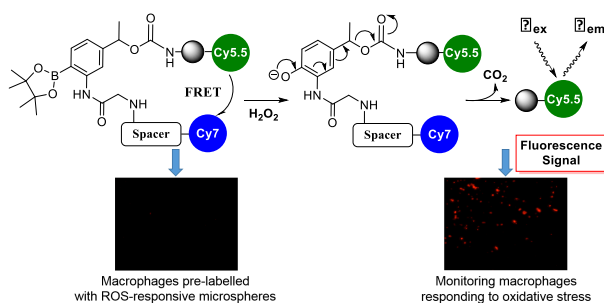
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# Synthesis and optimization of a reactive oxygen species responsive cellular delivery system

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Reactive oxygen species-responsive delivery systems for the detection of peroxides in live macrophages have been designed. The oxidative cleavage of a boronic ester to a phenol triggered by hydrogen peroxide followed by self-immolation of a ROS-sensitive cleavable linker via 1,6-elimination, allowed the disturbance of the fluorescence resonance energy transfer turning on the near-infrared fluorescence.